Localization of Aquaporin CHIP in the Human Eye: Implications in the Pathogenesis of Glaucoma and Other Disorders of Ocular Fluid Balance

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Purpose. The existence of integral membrane proteins that serve as selective water channels has been postulated to explain the movement of water across plasma membranes. Aquaporin CHIP (channel-forming integral membrane protein of 28 kd) is the first such channel to be characterized and is abundant in human erythrocytes and a variety of secretory and absorptive epithelia of the rat. Because disturbances in the movement of water characterize several ocular diseases, the distribution of CHIP in the human eye was studied.

Methods. Affinity-purified antibodies against purified CHIP protein were used for the indirect immunofluorescence localization of CHIP in human eye structures. Labeling was confirmed by immunoblot analyses of membrane preparations from eye structures.

Results. CHIP immunolabeling was found in the corneal endothelium, the lens epithelium, the nonpigmented epithelium of the ciliary process, the iris epithelium, and the endothelium of the trabecular meshwork and the canal of Schlemm.

Conclusions. The presence of CHIP water channels in the secretory and absorptive tissues of the human eye provides a mechanism for transcellular water movement and may be important for understanding diseases of the eye that involve excess or insufficient movement of ocular fluid such as glaucoma, cataracts, and Fuch's dystrophy. In addition, the existence of CHIP in the outflow pathways of the human eye provides a novel explanation for the movement of water out of the eye. Invest Ophthalmol Vis Sci. 1994;35:3867–3872.

Water balance in the body is primarily maintained by diffusional gradients restricted by plasma membranes. There are, however, specialized tissues in which the transport of water across plasma membranes is greater than simple diffusion predicts.[1] The discovery of the CHIP protein[2] and subsequent cloning[3] and expression[4] has recently enabled the first demonstration of a water-selective molecular channel. In addition, related cDNAs encoding functional water channels have been identified in diverse plant and mammalian species. This group of proteins has been referred to collectively as the "aquaporins."[5] Recognition of CHIP protein has provided a molecular explanation for the enhanced water permeability of erythrocytes and a number of rat epithelia, including kidney tubule, male reproductive tract, choroid plexus, bile duct, and ocular epithelia.[6–9]

Many components of the eye are not vascularized and must rely on the aqueous humor for nutritive support. The regulation of aqueous volume and pressure is complicated and is a function of fluid exiting and entering the eye. Defects in this regulation may lead to the increase in intraocular pressure associated with glaucoma. Most of the fluid entering the eye is actively secreted by a layer of nonpigmented epithelial cells that line the ciliary process. It is thought that these cells maintain a local ionic gradient that provides the force needed to move water from the extravascular space into the eye. Fluid leaves the eye primarily through the trabecular meshwork and then into Schlemm's canal and the venous system, and second-

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arily via the uveo-vortex route. The physical structure of the trabecular meshwork functions as a one-way valve that permits fluid to leave the eye while limiting backflow. To enter Schlemm's canal and leave the eye, the aqueous humor must cross a bilayer of endothelial cells. The uveo-vortex route involves the iris epithelium and ciliary muscle. Water enters these structures and leaves by way of their respective venous beds.

The possible importance of CHIP-mediated water movement in the eye has been suggested by the effects of mercurials on intraocular pressure. For example, the inhibition of aqueous outflow in the human eye by mercurials is not a result of the inhibition of glycolysis.[10] Although mercurials can affect tissues nonspecifically, they are also known to inhibit water transport specifically in a number of tissues, including the eye and kidney. In studies with CHIP, $HgCl_2$ inhibits water movement by a reversible reaction with a specific cysteine residue (Cys-189).[11]

The turnover of aqueous humor maintains and supports the functional components of the eye. The lens and cornea are thick, avascular structures dependent on water movement for providing nutrients and removing wastes. In addition, the physiological functions of the cornea and lens are dependent on their clarity. The transparency of the lens and cornea is contingent upon their state of hydration and appears to be maintained primarily by a surface layer of cells that have contact with the aqueous humor in the anterior chamber of the eye. It is hypothesized that the force required by these cells to move water is provided by osmotic gradients generated by the activity of NaK ATPase. The presence of CHIP then provides a mechanism for the passive movement of water in the direction of the resultant osmotic gradient.

Efficient movement of water is essential for the maintenance and proper functioning of the eye. Because numerous human diseases involve compromised ability to move water, we have undertaken this study to localize CHIP water channels in the human eye.

MATERIALS AND METHODS

Antibodies to CHIP

Immunization of rabbits with highly purified CHIP protein from erythrocytes was described previously.[2,5] Rabbit affinity-purified anti-CHIP IgG was used in immunofluorescence and immunoblot studies. Anti-CHIP reacts specifically with the 4 kd C-terminal cytoplasmic domain of CHIP.[2]

Human Eye Tissue

Human donor eyes were obtained from the Arizona Lions Eye Bank (Phoenix, AZ). For immunofluorescence microscopy, only adult eyes obtained less than 12 hours after death were studied. For immunoblotting, eyes (donor ages, 3 months to 80 years) were obtained between 12 and 48 hours after death, but comparisons of the various regions examined were made only with time- and donor-matched samples.

Tissue Isolation and Membrane Preparation

The ciliary process and the iris were carefully dissected from the anterior eye using Castroviejo scissors (Storz Instruments, St. Louis, MO). Trabecular meshwork was removed from the region between the scleral spur and Schwalbe's line by blunt dissection with an ocular foreign body instrument. Extra care was taken so as not to include corneal endothelium or iris tissue contaminants. Corneal epithelial and endothelial cells were scraped from corneas using a #15 scalpel. Lens epithelia were removed using forceps and Castroviejo scissors.

Tissue specimens were put into TME buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.5), were homogenized using a Polytron homogenizer (Brinkman Instruments, Westbury, NY), and, except for pigmented tissues, were centrifuged at 37,000g for 20 minutes. Pigmented tissues were centrifuged at 1800g for 10 minutes to remove pigment granules, and the supernatants were then centrifuged at 37,000g for 10 minutes. The membrane pellets were resuspended in TME buffer with a Duall homogenizer (VWR, Phoenix, AZ) and were centrifuged at 13,000g for 6 minutes. Pellets were resuspended in loading buffer (4% SDS, 125 mM Tris, 20% glycerol, 10% 2- mercaptoethanol) and were sonicated with a probe sonicator for 10 seconds just before loading.

Immunoblotting

Membrane preparations were electrophoresed into 12% polyacrylamide gels containing SDS. Proteins were blotted onto nitrocellulose using the Transblot system (Biorad, Hercules, CA). The blots were preincubated for 2 hours at room temperature in Tris-buffered saline, containing 5% nonfat powdered milk and 0.1% Tween (TBS-T), and were then probed with purified anti-CHIP IgG (1:500 dilution) overnight at 4°C. The blots were washed three times in TBS-T and were incubated for 2 hours at room temperature with goat anti-rabbit HRP-conjugated secondary antibody (1:1000 dilution) (Amersham, Arlington Heights, IL). Anti-CHIP labeling of the blots was visualized by chemiluminescence (Amersham, ECL) and exposure to Kodak X-OMAT (Eastman Kodak, Rochester, NY).

Immunofluorescence Microscopy of CHIP

Human eyes were segmentally dissected and quick frozen in a 2-methyl butane bath. The segments were imbedded in tissue-tek OCT (VHR, Philadelphia, PA)

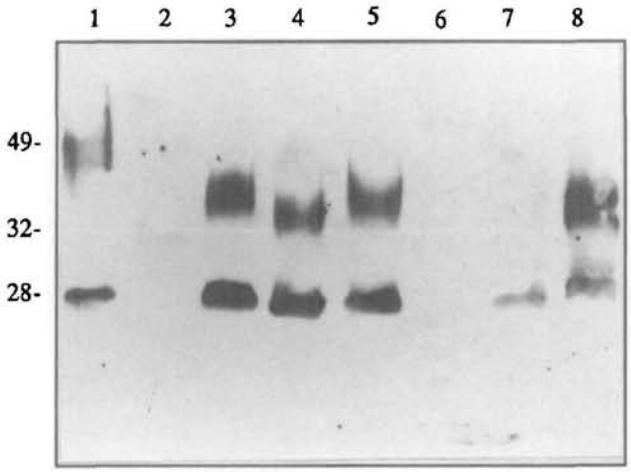

FIGURE 1. SDS-polyacrylamide gel electrophoresis and immunobloting of membranes isolated from various regions of the human eye. Dissection procedures and membrane preparation were as described in Materials and Methods. Samples were electrophoresed into 12% polyacrylamide gel slabs and were immunoblotted with purified anti-CHIP IgG. Visualization was by exposure of x-ray film to chemiluminescence. Erythrocyte membranes (1.1 μg) were a positive control (lane 1). The other samples and the amount of protein loaded were as follows: lane 2, retina (1.9 μg); lane 3, iris (0.4 μg); lane 4, lens (0.2 μg); lane 5, ciliary process (0.7 μg); lane 6, corneal epithelium (1.7 μg); lane 7, corneal endothelium (3 μg); and lane 8, trabecular meshwork (4.5 μg). The positions of the molecular size markers are indicated on the left ($\times 10^3$ kd).

and frozen at −80°C. Using a Hacker (Fairfield, NJ) cryostat microtome, 7- to 15-μm thick cryosections were obtained and fixed in 4% paraformaldehyde for 15 minutes. The cryosections were quenched twice in a 0.75% glycine and permeabilized in 0.1% Triton X-100. Cryosections were incubated for 1 hour with goat sera (1:200 dilution), washed twice in 2 × SSC (0.3 M NaCl, 0.03 M $NaC_6H_5O_7$) with 0.1% Triton X-100, and incubated overnight at 4°C with rabbit purified anti-CHIP IgG at a 1:500 dilution. Cryosections were washed three times and were incubated for 2 hours at room temperature with fluorescein-conjugated donkey anti-rabbit (Pierce, Rockford, IL) or with rhodamine-conjugated goat anti-rabbit secondary antibody (1:100 dilution) (Cappel, Durham, NC). Cryosections were washed in 2 × SSC with 0.1% Triton X-100 and mounted.

RESULTS

Western Blot Localization of Aquaporin CHIP in the Human Eye

Figure 1 shows the results of immunoblotting of membranes isolated from several human eye structures with purified anti-CHIP IgG. In lane 1, human erythrocyte membranes served as a positive control. The presence of CHIP is evidenced by the bands at M_r 28,000 and 49,000, which represent the nonglycosylated and glycosylated forms of CHIP, respectively. CHIP is also present in membranes prepared from isolated iris (lane 3), lens epithelium (lane 4), ciliary process (lane 5), corneal endothelium (lane 7), and trabecular meshwork–canal of Schlemm (lane 8). In these samples, the glycosylated form of CHIP has a greater mobility (M_r 32-40,000) than the erythrocyte membranes, suggesting that the pattern of glycosylation may be different for the ocular forms of CHIP. Membrane preparations from retina (lane 2) and from corneal epithelium (lane 6) are negative for CHIP. Based on the amount of protein loaded onto the gels, the iris, lens, and ciliary process have the highest content of CHIP per milligram of protein and, in fact, are comparable to the amount of CHIP present in erythrocyte membranes. The amount of CHIP per milligram of protein is less in the corneal endothelium and the trabecular region than in the other tissues.

Immunofluorescence Localization of Aquaporin CHIP in the Human Eye

Donor eyes from eight individuals were sectioned and were examined by immunofluorescence microscopy for the presence of CHIP. Specific labeling was only obtained with samples from the anterior chamber. In all cases, the specificity of labeling was verified by labeling in the absence of primary antibody, labeling with nonimmune rabbit sera, and labeling after preabsorption of the purified anti-CHIP IgG with a 50-fold excess of purified CHIP. Representative examples of immunofluorescence microscopy follow.

Cornea and Lens

Figure 2A shows that the monolayer of cells comprising the corneal endothelium is strongly labeled, whereas corneal epithelium, stromal tissue, and keratocytes are not. Labeling appeared to be present on both the apical and basolateral plasma membranes of these cells. Use of affinity-purified anti-CHIP was essential because preliminary studies with whole antisera to CHIP cross-reacted with structures of corneal epithelium (not shown). Similarly, Figure 2B shows that the epithelial cells of the anterior surface of the lens contained immunoreactive CHIP localized to both the apical and basolateral membranes, whereas labeling was not present in the lens fiber cells.

Ciliary Process

In Figure 3, the human ciliary process was examined by immunofluorescence microscopy to determine the cellular localization of CHIP. Figure 3A shows hematoxylin and eosin staining of the ciliary process with the cells of the nonpigmented epithelium (NPE) ex-

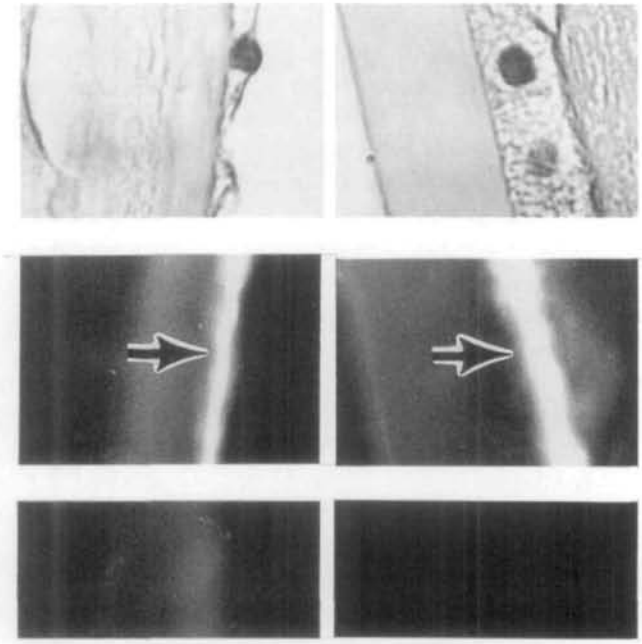

FIGURE 2. Immunofluorescence microscopy of the human cornea and lens after labeling with purified anti-CHIP IgG. The top panels are sections stained with hemotoxylin and eosin. The middle panels are tissue sections (7 to 15 μm) incubated with purified anti-CHIP IgG followed by incubation with rhodamine conjugated goat anti-rabbit IgG. The bottom panels show the nonspecific labeling after preincubation of anti-CHIP with CHIP. (*left*) Cornea (labeling of endothelial cells indicated by arrow). (*right*) Anterior lens (labeling of epithelial cells indicated by arrow). Original magnification, ×400.

ternal to the cells of the pigmented epithelium (PE). After labeling with anti-CHIP (Fig. 3C), there was abundant fluorescence in the NPE but not in the PE. At higher magnification (Fig. 3D), the labeling of NPE can be localized to the surface in contact with aqueous humor and the surface facing the pigmented epithelium. Preincubation of anti-CHIP with purified CHIP blocked most of the immunolabeling (Fig. 3B), but some fluorescence remained. In control studies in which both primary and secondary antibodies were omitted, this residual fluorescence was observed, showing that it was intrinsic or autofluorescent.

Outflow Pathways

The outflow of aqueous humor from the human eye takes place through the trabecular meshwork–canal of Schlemm (primary outflow pathway) and the iris (uveo-vortex route). The possibility that CHIP could be involved in the movement of water by these outflow pathways was assessed by immunofluorescence microscopy. On the posterior side of the iris (Fig. 4B), there was labeling with anti-CHIP of the pigmented epithelium that was blocked by preincubation with purified CHIP (Fig. 4C). Figure 4 also shows that the primary outflow pathway demonstrated immunoreactivity. Specifically, in the trabecular meshwork, the endothelial cells wrapping the beams (arrows in Fig. 4B) were labeled. Similarly, the juxtacanalicular region of the trabecular meshwork and the inner and outer walls of Schlemm's canal were specifically labeled. Preincubation of anti-CHIP with purified CHIP yielded only background fluorescence in all of these regions (Fig. 4C).

DISCUSSION

The trabecular meshwork, ciliary nonpigmented epithelium, and corneal endothelium of the human eye are known for their high permeability to water. However, the molecular mechanism of this permeability is poorly understood. The recent discovery of CHIP, a transmembrane water-selective channel, provides a possible molecular mechanism for the unusually high water permeability of these tissues. Using indirect immunofluorescence microscopic and immunoblot anal-

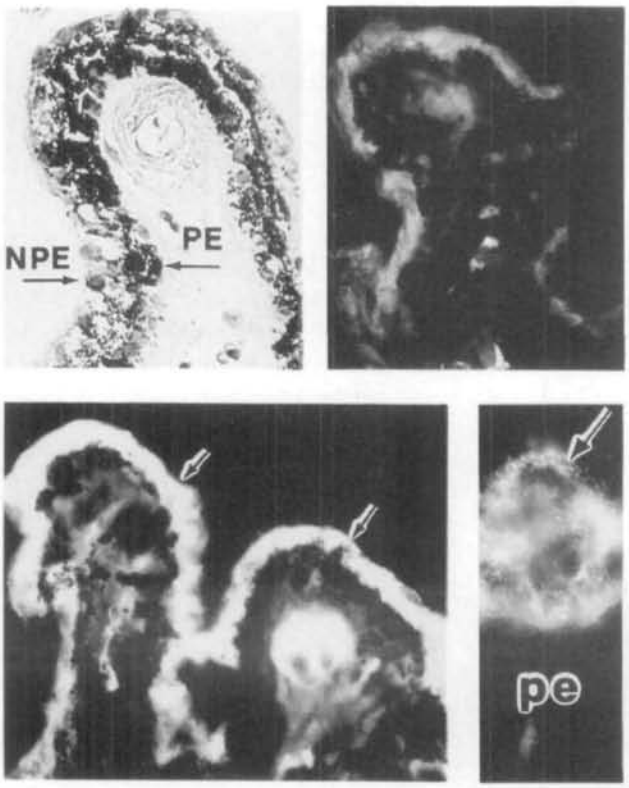

FIGURE 3. Immunofluoresence microscopy of the human ciliary process after labeling with anti-CHIP IgG. (*top left*) Hematoxylin and eosin-stained section showing the nonpigmented epithelium (NPE) and the pigmented epithelium (PE). (*top right*) Nonspecific labeling after preincubation of anti-CHIP with purified CHIP. (*bottom left*) Labeling of NPE (*arrows*). Original magnification, ×200. (*bottom right*) Labeling of NPE at higher magnification (*arrow*). Original magnification, ×1000.

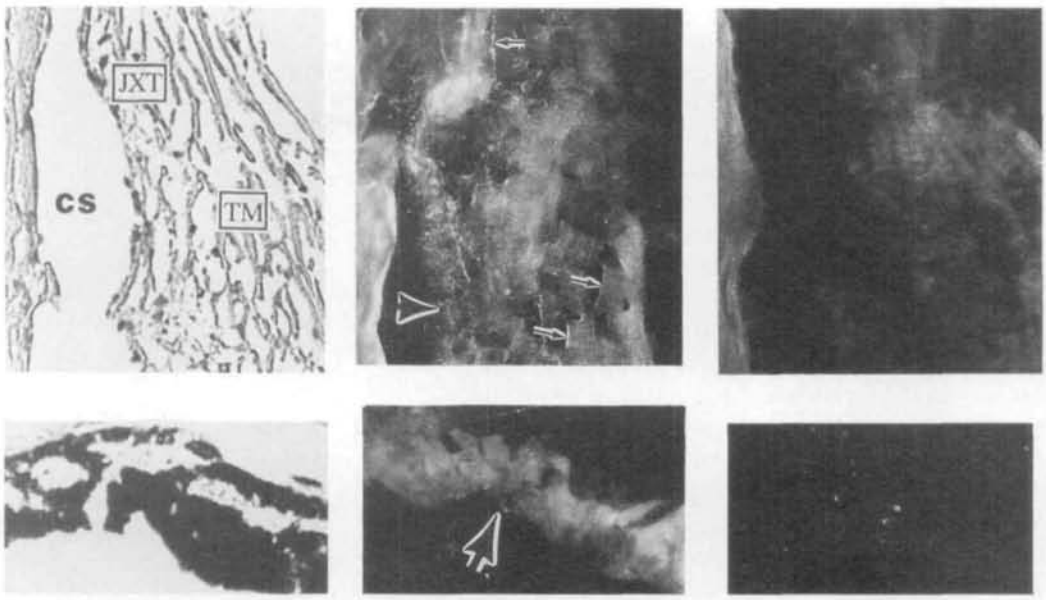

FIGURE 4. Immunofluorescence microscopy of the outflow pathways of the human eye after labeling with anti-CHIP IgG. The upper panels are photomicrographs of the trabecular meshwork region, and the lower panels are of the iris. (*left*) Hematoxylin and eosin-stained sections show the trabecular meshwork (TM), the canal of Schlemm (CS), the juxtacanalilicular region (JXT), and the iris. (*middle*) Labeling of the TM cells lining the trabecular beams (*small arrows*), the interior wall of canal of Schlemm (*large arrow*), the exterior wall, the juxtacanalicular area of the TM region, and the posterior pigmented epithelium (*arrow*) of the iris. (*right*) Nonspecific labeling after preincubation of anti-CHIP with CHIP. Original magnification, ×200.

yses, we have localized CHIP in the corneal endothelium, lens epithelium, and ciliary nonpigmented epithelium of the human eye. In addition, we have found CHIP in Schlemm's canal, the trabecular meshwork, and the iris, where it may play an important role in the movement of water out of the eye.

Our localization of CHIP in the human eye confirms recent findings of CHIP in the rat eye.[6,8] For example, using antibodies directed against human CHIP, positive immunofluorescence was found in the nonpigmented ciliary epithelium, the corneal endothelium, the lens epithelium, and the iris epithelium of the rat eye. In contrast to our present findings, however, CHIP could not be localized in the primary outflow region of the rat. Furthermore, instead of using membrane preparations from specific regions of the eye, membranes were prepared from the entire anterior segment. Therefore, the anterior segment was shown to contain an immunoreactive 28-kd band, but its origin could not be determined. In addition, other studies using in situ hybridization have indicated that mRNA encoding CHIP may be present in the nonpigmented epithelium and corneal endothelium in the rat eye.

Aquaporin CHIP proteins form open, water-selective pores that permit the rapid movement of water across the plasma membrane in the direction of the prevailing osmotic gradient.[5] Thus far, in many of the tissues in which CHIP has been identified, there are high levels of NaK ATPase. The activity of this enzyme is required for generating the osmotic gradients needed to allow CHIP to move water across the membrane at rates greater than simple diffusion would predict. This is especially true for most of the ocular tissues in which we have found CHIP. Therefore, the ciliary process, corneal endothelium, and lens epithelium all contain high levels of NaK ATPase.[12] The importance of this enzyme for generating the force needed to maintain the flow of water in the eye is evidenced by the effects of ouabain, which has been found to decrease substantially the formation of aqueous and to cause hydration of the cornea and lens. In addition to finding CHIP in tissues known for their high levels of NaK ATPase, we also found CHIP in ocular tissues that do not contain high levels of this enzyme. Thus, CHIP was present in cells of the trabecular meshwork, the endothelium of the canal of Schlemm, and the epithelium of the iris. In these tissues, it is possible that the force needed to move water through the channels is provided by a gradient of hydrostatic pressure. Such a gradient is thought to be important in maintaining retinal attachment. However, we did not observe anti-CHIP immunofluorescence in the cells of the retinal pigment epithelium. These studies, which were complicated by the presence of pigment granules and autofluorescence of ret-

inal tissues, suggested that either CHIP is not present or that a homologue of CHIP may be functioning in these cells.

Two possible roles for CHIP are in the movement of water in the eye in aqueous humor production by cells of the ciliary process and in outflow by way of the trabecular meshwork. A third role for CHIP could involve the movement of water needed to maintain the state of hydration of the refracting surfaces of the eye, that is, the cornea and lens. This is important because, to maintain clarity, the latticework of proteins that make up the cornea must be properly spaced, which, in turn, depends on the amount of water present. Similarly, maintaining the transparency of the lens depends on regulating the water content of fiber cells and their interstitial fluid. The presence of CHIP in the epithelial cells lining the lens and in the endothelium of the cornea provides a pathway by which water could rapidly enter and leave these tissues. Moreover, recent experiments with antisense probes indicate that CHIP RNA accounts for the endogenous water-channel transcripts in cultured bovine corneal endothelium.[9]

Our findings of CHIP water channels in the human eye provide a mechanism to help explain earlier models of water movement in the structures in which we have detected CHIP. Specifically, a model for the transport of water out of the cornea was proposed to be dependent on the activity of ionic pumps in the corneal endothelium.[13] This model, known as the pump-leak hypothesis, asserts that the active movement of fluid out of the cornea equals the movement of fluid leaking into the cornea. The presence of CHIP in the corneal endothelium would facilitate this movement of fluid.

The presence of aquaporin CHIP in several regions of the human eye emphasizes the importance of the efficient movement of ocular fluids. Several diseases of the human eye have been associated with the inability to maintain normal hydration of the tissue, such as Fuch's dystrophy of the cornea and cataracts of the lens.[12] An understanding of the role that CHIP plays in the movement of water into the lens and cornea and in the regulation of aqueous flow may improve our understanding of these diseases in humans and lead to novel therapeutic strategies.

Key Words

corneal endothelium, ciliary epithelium, lens epithelium, iris epithelium, trabecular meshwork

Acknowledgments

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